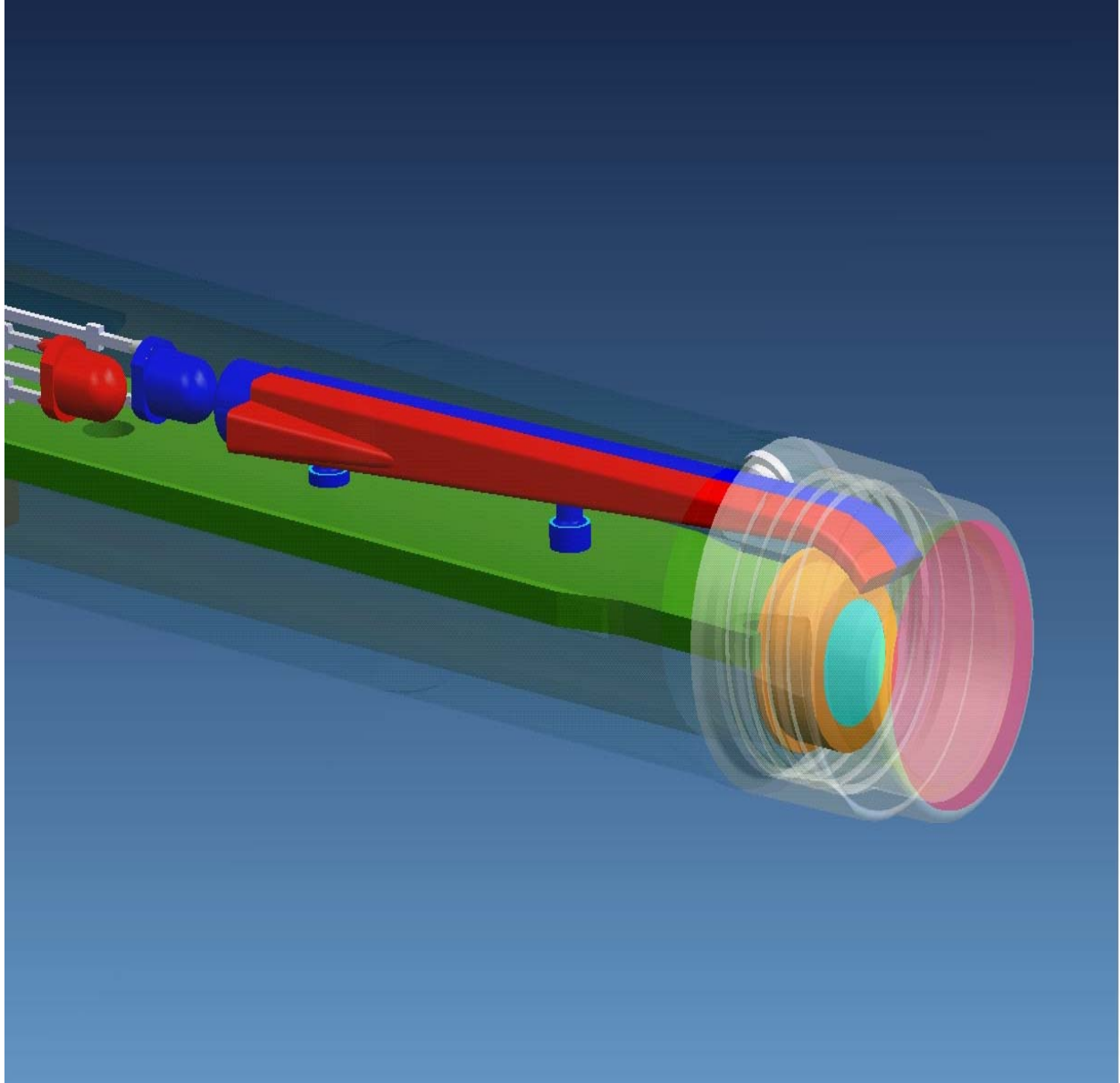# Report on the Validation of Proposed EPA Method 360.3 (Luminescence) for the Measurement of Dissolved Oxygen in Water and Wastewater

Report on the Validation of Proposed EPA Method 360.3 (Luminescence) for the Measurement of Dissolved Oxygen in Water and Wastewater

Acknowledgements

Disclaimer

This report has been reviewed and approved for distribution by Hach Company. The mention of contributor names outside of Hach Company does not constitute their endorsement of the luminescence method or their recommendation for use.

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Section 1
Executive Summary

Dissolved oxygen (DO) is one of the most important of the dissolved gases found in water. In natural waters, DO exists in a dynamic equilibrium controlled by biochemical depletion and oxygenation through atmospheric diffusion, aeration, and photosynthesis. As a result, bacterial populations proliferate and provide a key input up the food chain. However, dissolved oxygen is subject to detrimental fluxes when a catastrophic event occurs such as the discharge of organic waste into natural waters. Depending on the severity of the insult, DO may be depleted to the point where higher trophic organisms such as macro invertebrates and fish are killed off.

In wastewater treatment, organic-based sewage is degraded under controlled aerobic conditions. Failure to maintain adequate supplies of DO result in anaerobic conditions that lead to offensive and corrosive sulfides. Excessive aeration, on the other hand, is wasteful and drives up unnecessary operational costs. Prudent monitoring of DO is essential for assessing environmental risk in natural waters and for optimal wastewater treatment performance and regulatory compliance.

As a result, precision and accuracy of the DO measurement becomes a critical issue of interest - not only for estimating the degree of water quality or purification, but in calculating industry discharge loading costs from public owned treatment work (POTW) facilities. And while the two regulatory EPA methods of Winkler titration (360.2) and membrane probe (360.1) have been in use for 30 or more years, they often fall short in delivering interference-free readings that are accurate and precise.

This report details the results of an interlaboratory validation study for the development of Proposed EPA Method 360.3 (Luminescence) for the Measurement of Dissolved Oxygen in Water and Wastewater.   Twelve wastewater facilities, representative of the United States POTW wastewater industry, analyzed DO reference water samples and wastewater matrices by the two EPA approved methods and luminescence. Results of the study clearly demonstrate that the luminescence procedure to be more accurate and precise than either the Winkler titration or the membrane probe techniques currently used for reporting DO. The primary benefit of the luminescence method is better performance with respect to accuracy and precision, regardless of wastewater matrix (influent-to-treatment and final effluent). Additional benefits to the luminescence method over EPA Methods 360.1 and 360.2 include elimination of toxic and or hazardous chemicals, no membrane to change, simplicity of use, and increased sample-throughput.

Section 2
Background

Overview of Dissolved Oxygen

Dissolved oxygen (DO) is without question the most important of the dissolved gases found in water. In natural waters DO exists in a dynamic equilibrium controlled by biochemical depletion and oxygenation through atmospheric diffusion, aeration, and photosynthesis. That is, as microbial growth in the water column degrades organic matter, oxygen is consumed. Oxygen is re-supplied through atmospheric diffusion, aeration, and photosynthesis. As a result, bacterial populations proliferate and provide a key input up the food chain.

Dissolved oxygen equilibrium is subject to detrimental fluxes when a catastrophic event occurs such as organic waste being discharged to the natural water body. An immediate depletion in DO results in an anoxic environment. Depending on the severity of the insult, DO may be depleted to the point where higher trophic organisms such as macro invertebrates and fish are killed off.

In wastewater, organic-based sewage is degraded under controlled aerobic conditions. Failure to maintain adequate supplies of DO result in anaerobic conditions that lead to offensive and corrosive sulfides. Excessive aeration on the other hand is wasteful and drives up unnecessary operational costs. When DO is reported in aeration basins and outfalls or used to derive the biochemical oxygen demand from wastewater, it becomes a regulatory tool. Thus, prudent monitoring of DO is essential for assessing environmental risk in natural waters and for optimal wastewater treatment performance and regulatory compliance.

Historical Development of Dissolved Oxygen Measurements

The Winkler titration procedure (Winkler, 1888, 1911, and 1912) is the first recognized method (*Standard Methods*, 1st ed., 1912) for elucidating the process of oxygen consumption in polluted water. However, the Winkler method is well known to exhibit inaccurate results with some polluted water samples.

The membrane electrode procedure was first reported by Carritt and Kanwisher and Mancy in 1959 and Mancy et al., in 1962. However, not until 1975 did membrane electrodes become an accepted method (*Standard Methods*, 14th ed.). And while membrane electrodes revolutionized the process of measuring DO, they are not without their limitations. The reduction reaction intrinsic to the response mechanism of membrane electrodes consumes oxygen. A second and more burdensome problem is the instability of calibration. As a result frequent calibration may be required. This stems from the fact that the magnitude of cathodic current is flux dependent. That is, membrane electrodes are designed so that oxygen diffusion across the gas-permeable membrane is rate limiting, thereby controlling the flux of oxygen to the electrode. Membrane fouling such as oil and grease and other interfacial barriers can alter the rate

of diffusion across the membrane, thus altering the calibration function for the electrode. Another potential limitation to membrane electrodes is the depletion and degradation, and in some cases poisoning, of the internal electrolyte solution and electrodes. Dissolved oxygen crosses the membrane, dissolves into the electrolyte solution, and diffuses to the cathode where oxygen reduction takes place. Any changes to the solution or electrode can adversely affect calibration and performance. To illustrate, calibration of the membrane electrode is based on a single measured data-point with the assumption the slope of all data points in the measurement range of the electrode passes through the origin (0,0). Since the measured data point is most often the value from a water-saturated air (i.e., 7 to 9 ppm) calibration technique (*Standard Methods*, 20th ed.) and the value compared to a Winkler titration, the low end of the assumed calibration range (1 ppm) is rarely if ever verified. Over time, with continued deteriation of the three main determinate variables (membrane condition, electrolyte solution purity, electrode integrity) and while the upper end of the calibration may not change or change slightly, the lower measurable end is not verified.

New Dissolved Oxygen Determinant

The application of oxygen quenching luminophores has gained substantial prominence in the last 19 years (Bergman, 1986; Lubbers, 1992; Gruber et al., 1993; Weigl et al., 1994; Klimant, Meyer, and Kuhl, 1995; Reininger, Trettnak, and Grubber et al., 1996; Chang and Arnold, 1999) due to their increased sensitivity to oxygen, resilience to most interferences associated with the Winkler titration procedure and membrane electrodes, and are non-oxygen consuming, which makes them less sensitive to flow velocity. Their intrinsic properties are such that the quenching of luminescence emission can be accurately and precisely measured with few if any interferences.

New Method Justification

Dissolved oxygen (DO) is one of the most sought out measurement in the operation of wastewater treatment plants. Consequently, precision and accuracy of the determinant to measure DO becomes a critical issue of interest - not only for estimating the degree of water quality or purification, but in calculating industry discharge loading costs from public owned treatment work (POTW) facilities. And while the two EPA methods (360.1 and 360.2) have been in use for 30 or more years, they often fall short in delivering interference-free readings that are accurate and precise.

Winkler Method (EPA Method 360.2)

The Winkler method is a destructive chemical titration technique subject to numerous interferences (i.e., oxidizing and reducing agents, nitrite ion, ferrous and ferric iron, suspended solids, and organic matter). Aqueous samples are treated with manganous sulfate, potassium hydroxide, and potassium iodide to form manganous hydroxide, $Mn(OH)_2$. Oxygen in the sample reacts with the Mn(II) species giving Mn(III).

Due to instability, Mn(III) further reacts with another O_2 molecule to form the Mn(IV) species. Upon acidification, $MnO(OH)_2$ forms manganic sulfate and acts as an oxidizing agent to release free iodine (I_2). Iodine, which is stoichiometrically equivalent to the DO in the sample, is titrated with sodium thiosulfate or phenylarsine oxide to its starch indicator endpoint. The Winkler method is subject to numerous interferences such as the presence of nitrite ion, ferrous and ferric iron, suspended solids, and organic matter (*Standard Methods*, 20th ed.).

Membrane Electrode Method (EPA Method 360.1)

Membrane electrodes have a thin organic membrane covering a layer of electrolyte and two metallic electrodes. Oxygen diffuses through the membrane and is electrochemically reduced at the cathode. There is a carefully fixed voltage between the cathode and anode so that only oxygen is reduced. The greater the oxygen partial pressure, the more oxygen diffuses through the membrane in a given time. This results in a current that is proportional to the oxygen in the sample. Temperature sensors built into the probe on some advanced measurement systems allow compensation for the membrane and sample temperatures, which affect diffusion speed and solubility. The meter uses cathodic current, sample temperature, barometric pressure, and salinity information to calculate the dissolved oxygen content of the sample. The reductive voltage is supplied either electronically by the meter (potentiometric oxygen electrode) or selective dissimilar metals may be used so that the correct voltage is generated between them (galvanic electrode).   Due to interfacial dynamics at the membrane-sample interface (i.e., the consumption of oxygen and diffusion dependent), sufficient interfacial turbulence is necessary to maintain accuracy and precision of DO analysis. Additionally, membrane electrode performance is subject to various types of inorganic salts, reactive gases, and their concentrations.

Luminescence Method (Proposed EPA Method 360.3)

The proposed luminescence sensor method is based upon dynamic fluorescence quenching of a luminophore (luminescent dye molecule) by oxygen. A thin film of the luminophore is entrapped in an oxygen permeable polymer and undergoes excitation by absorption of photons being produced from an LED light source. Upon relaxation (dissipation of excitation energy), light of lower energy i.e. longer wavelength, is emitted (luminescence). The measured magnitude of steady state luminescence (intensity) and/or average relaxation time (luminescence lifetime) is inversely proportional to the concentration of oxygen and thus, the signal to noise ratio of the measurement increases with decreasing DO concentration.

Method Summary

The new method has a luminescence sensor that consists of an indicator dye layer immobilized at the surface of an optically transductive support material. The indicator layer consists of an oxygen sensitive luminophore. A light emitting diode (LED) provides incident light required to excite the luminophore. The resulting dynamic lifetime of the excited luminophore is measured and equated to DO concentration.

Applicable Matrices

This method is amenable to all water and wastewater matrices.

Measured analyte
Oxygen   CAS Registry Number   7782-44-7

Interferences

There are no known interferences at normal wastewater concentrations that interfere with DO detection and quantification.

**Section 3
Objectives**

Validation Study Purpose

The purpose of this study is to validate the proposed luminescence method (Method 360.3) for EPA regulatory acceptance and approval. Validation is the process to establish EPA quality assurance acceptance criteria that are used in method promulgation and demonstration of method equivalence to that of the EPA reference method.

Study Design

The study design for this validation was based on the *Protocol for EPA Approval of New Methods for Organic and Inorganic Analytes in Wastewater and Drinking Water* (EPA-821-B-98-003, March 1999), principles outlined in *Guidance for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* (JAOAC 72 No.4, 1989), *Use of Statistics to Develop and Evaluate Analytical Methods* (AOAC-International), and ASTM Standard D-2777 (ASTM). The intent of validation is to allow all regulated entities and laboratories to apply a new method to a single sample matrix type in a single industry. Since wastewater is considered a single matrix type and POTWs (Publicly Owned Treatment Works) represent a single industry, a multiple laboratory validation facilitates nationwide use of a new method for the analysis of wastewater.

For this study, influent-to-treatment and final effluent wastewater are considered two different matrices and are validated separately in establishing method equivalence with that of the EPA reference method (Winkler, EPA Method 360.2) and the alternate method (Membrane, EPA 360.1). Quality assurance acceptance criteria from the analysis of reference water are shared for both influent-to-treatment and final effluent wastewater matrices.

The validation for both matrices is performed through the analysis of reference water samples that generated a method detection limit (MDL), a method limit (ML), and upper and lower limits of recovery, upper limit of precision, and a side-by-side comparison of each determinant with influent-to-treatment and final effluent wastewater samples. Due to the restraints and limitations in spiking DO in matrix samples, matrix spike and matrix spike duplicate results are not generated. Additionally, calibration is performed with a single-point (water-saturated air) and assumes a straight-line through the origin over the typical wastewater DO reporting range. Calibration verification is performed with air-saturated water.

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Section 4
Study Management

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Organization

The organization responsible for the management of this study is Hach Company. Hach Company, on behalf of POTWs nationwide is conducting the validation study and submitting the results to EPA for Tier approval.

Facility and Laboratory Participants

Facility Name	Location
City of Livermore WWTP	Livermore, California
East Bay Municipal Utility District	Oakland, California
Sand Creek Water Reuse Facility	Aurora, Colorado
City of Fort Collins WWTP	Fort Collins, Colorado
City of Loveland WWTP	Loveland, Colorado
Main Street WWTP	Gainesville, Florida
Hach Company (in-house)	Loveland, Colorado
Metro St. Louis WWTP	St. Louis, Missouri
Trinity River Authority WWTP	Dallas, Texas
Nacogdoches WWTP	Nacogdoches, Texas
City of Byron WWTP	Byron, Illinois
City of Longview WWTP	Longview, Texas
Hornsby Bend WWTP	Austin, Texas

Study Schedule

The interlaboratory validation study schedule was designed in three phases. The first phase was for the laboratories to prepare air-saturated water and demonstrate they could obtain DO recoveries that were inconsistent with in-house study results. Phase I was conducted during the week of July 19, 2004.

Phase II of the study was laboratory receipt of reference water samples that were prepared by Hach Company, and the collection and analysis of influent-to-treatment and final effluent wastewater samples. The reference water samples were analyzed for the determination of IPR, MDL, and ML values used in the developing QC acceptance criteria. Analysis of wastewater samples was used to evaluate method performance comparisons and correlation. Phase II occurred during the weeks of July 26 and August 2, 2004.

Phase III was the collection and statistical analysis of the data received from each of the participating laboratories. Data was reviewed for completeness, evaluated for outliers, and statistically processed for validation report preparation. Phase III occurred during the weeks of August 9 through August 20, 2004.

Section 5
Development of Quality Control Acceptance Criteria

This section of the report presents details of the statistical analyses used to develop the Quality Control (QC) acceptance criteria for the proposed Method 360.3. Data generated from this study and from Hach Company's in-house validation study are used for the development of these criteria.

Study data that were used to develop the QC acceptance criteria consisted of results from IPRs at two concentration ranges that represent the analytical testing range for the determination of BOD from Standard Methods, 20th ed. and results from MDL data.

An advantage of using data from both the in-house study and interlaboratory studies was based on results from both a breadth and depth of laboratory analyses. Use of data from the in-house validation study provides results from 52 IPR analyses at the upper analytical testing range, 44 IPR analyses at the lower analytical testing range, and 77 MDL analyses; use of data from the inter-laboratory study provides IPR results from 7 laboratories and MDL results from 6 laboratories.

The basic design of the interlaboratory validation study is described in detail in the Hach Company Proposed Study Plan (Hach Company, 2003) and the Validation Study Protocol (Hach Company, 2004).

Data Validation and Statistical Analysis

Results from each of the interlaboratory validation laboratories were first compared to results from an earlier in-house IPR validation study to determine if the reference water samples (IPR_{low}) had been compromised during shipment and if any laboratories were systematically biased. Recovery results from any IPR reference water samples that were ± 3 standard deviations from the in-house IPR mean were rejected as sample integrity compromised. To determine systematic bias, the median percent recovery of results from all laboratories (in-house and interlaboratory) that passed the reference water sample integrity test was calculated. Then the percent recovery values of each of the four IPR samples were then analyzed by an extreme rank test. If all the laboratories were equivalent, then theoretically the ranks of each laboratory relative to the other laboratories would be random, and the summed ranks would be equal. The extreme rank test determines if the summed rank of any laboratory is significantly different from those of the other laboratories. The extreme rank test is based on the work of *Youden in Ranking Laboratories by Round Robin Tests* (1963).

To determine if the MDL reference water samples had been compromised during shipment, individual replicate DO results from each laboratory were compared to the theoretical DO. Results greater than three times the theoretical DO were determined compromised and thus, eliminated from the study.

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To calculate the relative percent difference (RPD) between the two EPA methods and luminescence for the influent and effluent matrices, the absolute difference between the 2 determinant DO measurements was divided by the average of the 2 measurements and multiplied by 100.

Initial Precision and Recovery Statistics

Quality control acceptance criteria are calculated using the average percent recovery and the standard deviation of recovery from the IPR tests on four aliquots of the reference matrix in the eight laboratories, as follows:

(1) Calculate the average percent recovery (X_{avg}) for each analyte based on all data points from all laboratories, the between-laboratory standard deviation (s_b) of the mean results for each of the three laboratories (standard deviation of the eight lab means), and the pooled $X_{(lab\ 1)}$, $X_{(lab\ 2)}$, $X_{(lab\ 3)}$ within-laboratory standard deviation (s_w). s_w is calculated as the square root of the mean of all within-laboratory variances.

(2) To calculate a 95% confidence interval for precision, the RSD (computed as s_w divided by X) is multiplied by the square root of a 95th percentile F value with 3 degrees of freedom in the numerator and $m(n - 1)$ degrees of freedom in the denominator, where m = the number of laboratories, and n is the number of data points per laboratory. For example, the resulting multiplier on the RSD for three laboratories and five data points per laboratory will then be 1.9, and the QC acceptance criterion for precision in the IPR test (RSD_{max}).

(3) To calculate QC acceptance criteria for recovery, the combined standard deviation for interlaboratory variability and estimation of the mean (s_c) as:

$$S_c = \sqrt{\left(1 + \frac{1}{4}\right) S_b^2 + \left(\frac{1}{4} - \frac{1}{n}\right) S_w^2}$$

where m = the number of laboratories, and n = the number of data points per laboratory.

(4) Calculate the QC acceptance criteria for recovery in the IPR test by constructing a ± 3.2 s_c window around the average percent recovery (X_{avg}, where 3.2 is the 97.5th percentile Student's t value for 3 degrees of freedom (an estimated degrees of freedom based on the variance ratios observed with EPA Method 1625).

An example of the actual computations to derive IPR QC acceptance criteria is found in Appendix A.

Method Detection Limits and Minimum Levels Statistics

This procedure involves analyzing seven replicate aliquots of reference water at a concentration within one to five times the estimated MDL (0.07 - 0.09 mg/L). The seven aliquots are then carried through the entire analytical process, and the standard deviation of the seven replicate determinations is calculated. The standard deviation is multiplied by 3.143 (the Student's t value at 6 degrees of freedom) to form the MDL.

A pooled MDL is calculated from m individual laboratory MDLs by computing the square root of the mean of the squares of the individual MDLs and multiplying the result by a ratio of t-values to adjust for the increased degrees of freedom.

$$MDL_{pooled} = \frac{\sqrt{d_1\left(\frac{MDL(Lab1)}{t(0.99, d_1)}\right)^2 + d_2\left(\frac{MDL(Lab1)}{t(0.99, d_2)}\right)^2 + d_3\left(\frac{MDL(Lab1)}{t(0.99, d_3)}\right)^2}}{d_1 + d_2 + \ldots d_{\ldots}} \, t_{(0.99, d_1 + d_2 + d_m)},$$

where m = the number of laboratories, and d_i = one less than the number of replicates used by lab i to derive the MDL.

The ML is established by multiplying the pooled MDL by 3.18 and rounding to the number nearest to (1, 2, or 5) x $10n$, where n is positive or negative integer. The purpose of rounding is to allow instrument calibration at a concentration equivalent to the ML without the use of unwieldy numbers. The use of 3.18 results in an overall standard deviation multiplier of 10, which is consistent with the American Chemical Society's (ACS) limit of quantification (LOQ) (P.S. Porter et al., *Environ. Sci. Technol.*, 22, 1988).

An example of the actual computations to derive MDL QC acceptance criteria is found in Appendix A.

Section 6
Results and Discussion

Initial Precision and Recovery

Seven of the twelve interlaboratory validation laboratories and the single in-house validation study laboratory were used to develop the IPR_{low} QC acceptance criteria. Three of the 12 interlaboratory validation laboratories were determined to have received reference water samples where the water seal had been lost. The fourth laboratory did not follow the validation study protocol for measuring DO. Therefore these 4 laboratories were excluded from this part of the study. Statistical results from the 8 included laboratories produced zero outliers and showed that all these laboratories to be equivalent.

Ten of the twelve interlaboratory validation laboratories and the single in-house validation study laboratory were used to develop the IPR_{high} QC acceptance criteria. One of the 12 laboratories failed to report results for air-saturated water (IPR_{high}) samples and the remaining laboratory did not follow the validation study protocol for measuring DO. Therefore, both of these laboratories were excluded from this part of the study. The in-house laboratory data was produced from an earlier study and pooled with interlaboratory calibration verification data. Each validation laboratory produced two data points for the IPR calculation, whereas the in-house laboratory produced 4 data points. To pool the data from both the in-house and interlaboratory validation laboratories, the total number of data points where averaged across the laboratories, yielding 3 data points per laboratory set.

Results are summarized in Table 1 below. Individual laboratory results and the statistical analysis of these data are presented in Tables 2 through 7. The IPR results were compared to the respective QC acceptance criteria. For the IPR_{low} test (1.72 mg/L –1.74 mg/L), all mean percent values were within the calculated IPR recovery criterion (95.4% -104%) and four of the 18 IPR sets fell outside the precision criterion (1.75%). Realistically, three of these precision failures (2.03%, 1.89%, and 1.77%) should not be considered "failed" as the intrinsic properties of the luminescence technology produces highly precise and accurate DO measurements that are outside the norm of typical EPA regulatory precision limits. Therefore, the calculated 95th upper limit precision criteria of 1.75% should be expanded to reflect reasonable data quality objectives.

For the IPR_{high} test (7.22 mg/L – 9.23 mg/L), all mean percent values were within the IPR recovery criterion (96.6% - 104%) and three of the 23 IPR sets fell outside the precision criterion (1.110%). It should be noted that the three failed IPR sets (2.62%, 1.84%, and 1.20%) were based on the standard deviation of two samples each. However, based on the sampling population and QC acceptance criteria of the IPR_{low} study, it is unlikely that the IPR_{high} study would have significantly different 95th upper limit precision criteria from an increased IPR set of four samples. Therefore, like with the IPR_{low} study, the calculated 95th upper limit precision criteria of 1.10% should be expanded to reflect reasonable data quality objectives.

Table 1. QC Acceptance Criteria for Proposed Luminescence Method

IPR Range	IPR Conc. (mg/L)	97.5% Lower Limit of Recovery (%)	97.5% Upper Limit of Recovery (%)	95% Upper Limit of Precision (%)
Low	1.72 – 1.74	95.4	104	1.75
High	7.22 – 9.23	96.2	104	1.10

Table 2. Initial Precision and Recovery, Low Concentration (1.72 mg/L –1.74 mg/L)

Lab No.	% Rec. IPR #1	% Rec. IPR #2	% Rec. IPR #3	% Rec. IPR #4	Mean % Rec.	Standard Deviation
1	101	100	101	100	100	0.33
5	96.0	97.1	97.1	96.6	96.7	0.55
7	102	102	102	101	102	0.72
8	104	98.9	103	99.4	101	2.68*
9	98.9	98.9	100.0	98.9	99.1	0.57
10	101	102	102	102	102	0.29
11	99.4	100	101	101	100	0.86
13	101	99.4	98.3	98.3	99.1	1.09
13	101	99.4	98.3	98.3	99.1	1.09
13	98.9	97.7	97.1	97.1	97.7	0.81
13	101	99.4	98.3	97.7	99.0	1.27
13	102	99.4	98.3	97.7	99.3	1.77*
13	102	101	98.9	100	101	1.48
13	98.3	97.7	98.3	97.7	98.0	0.33
13	104	101	100	99.4	101	2.03*
13	101	98.3	97.7	96.0	98.1	1.89*
13	101	97.1	98.3	98.3	98.7	1.71
13	100	98.9	98.9	97.7	98.9	0.93
Mean % Recovery Across Labs					99.5	
Std. Deviation of Mean % Recovery Across Labs					1.43	
Mean % Std. Deviation Across Labs						1.31
% Std. Deviation of Variability and Estimation of Mean						1.96

* Result that failed the precision specification

Table 3. Initial Precision and Recovery (Low Concentration) Upper and Lower Limits of Recovery

97.5% Lower Limit (%)	97.5% Upper Limit (%)	No. of Mean % Rec. Values Below Lower Limit	No. of Mean % Rec. Values Above Limit
95.4	104	0	0

Table 4. Initial Precision and Recovery (Low Concentration) Upper Limit for Precision

95% Upper Limit (%)	No. of Std. Deviation Values Above 95% Upper Limit
1.75	4

Table 5. Initial Precision and Recovery, High Concentration (Air-saturated Water 7.22 mg/L – 9.23 mg/L)

Lab No.	% Rec. IPR #1	% Rec. IPR #2	% Rec. IPR #3	% Rec. IPR #4	Mean % Rec.	Standard Deviation
1	96.7	96.0	ND	ND	96.4	0.49
2	98.2	98.9	ND	ND	98.6	0.49
3	101	99.2	ND	ND	100	1.20*
5	96.0	97.1	ND	ND	96.6	0.81
6	100	101	ND	ND	101	0.35
7	101	98.4	ND	ND	99.7	1.84*
8	100	99.6	ND	ND	100	0.71
9	107	104	ND	ND	106	2.62*
10	98.6	99.8	ND	ND	99.2	0.85
11	102	102	ND	ND	102	0.28
13	101	100	100	100	100	0.17
13	101	101	101	101	101	0.20
13	99.9	100	100	100	100	0.28
13	100	100	100	101	100	0.18
13	101	101	101	101	101	0.11
13	101	101	101	101	101	0.13
13	101	101	101	101	101	0.13
13	101	101	101	101	101	0.18
13	101	101	100	100	101	0.26
13	101	101	101	101	101	0.11
13	101	101	101	101	101	0.08
13	101	100	101	101	101	0.24
13	100	100	101.0	101	101	0.29
Mean % Recovery Across Labs					100	
Std. Deviation of Mean % Recovery Across Labs					1.74	
Mean % Std. Deviation Across Labs						0.83
% Std. Deviation of Variability and Estimation of Mean						1.76

* Result that failed the precision specification
ND - not measured

Table 6. Initial Precision and Recovery (High Concentration) Upper and Lower Limits of Recovery

97.5% Lower Limit (%)	97.5% Upper Limit (%)	No. of Mean % Rec. Values Below Lower Limit	No. of Mean % Rec. Values Above Limit
96.6	104	0	0

Table 7. Initial Precision and Recovery (High Concentration) Upper Limit for Precision

95% Upper Limit (%)	No. of Std. Deviation Values Above 95% Upper Limit
1.10	3

Method Detection Limit and Method Limit

Six of the twelve interlaboratory validation laboratories and the single in-house validation study laboratory were used to develop the MDL and ML QC acceptance criteria. Five of the interlaboratory validation laboratories were determined to have received reference water samples where the water seal had been lost or that sensor-conditioning step performed immediately prior to MDL measurements was unsatisfactory, invalidating the associated DO measurements. The remaining laboratory did not follow the validation study protocol for measuring DO. A total of 6 laboratories were excluded from this part of the study.

Results of the 17 MDL tests are summarized in Tables 8A through 8C, and Table 9 below. Tables 8A through 8C report values from the individual analyses of seven MDL reference water samples from each laboratory, the mean concentration of each set of replicates, the standard deviation of the seven results, and their calculated MDL and ML values. Table 9 reports the pooled MDL and ML values for the in-house study, interlaboratory study, and the combination of the two studies.

The first MDL study by the in-house validation laboratory yielded a pooled MDL of 0.02 mg/L and a resultant ML of 0.07 mg/L. A Ph.D. chemist performed this study with over 20 years' experience in analytical chemistry and instrumentation. As expected, the results are representative of the skills and laboratory technique of a chemist at the Ph.D. level.

The second MDL study performed by the interlaboratory validation laboratories yielded a pooled MDL of 0.06 mg/L and a resultant ML of 0.18 mg/L. Six different POTW laboratories with a wide range of experience and laboratory technique produced these results.

From these two MDL studies, one easily concludes that skill level and laboratory technique from the in-house study reflect the lower MDL and resultant ML values. And while it is appropriate for the proposed luminescence method to be representative of a better performing laboratory, the MDL should also reflect the laboratory technique and skills from personnel most likely to perform the procedure. Therefore, the results from both the in-house validation and interlaboratory validation studies are pooled to determine the final MDL and ML (0.05 mg/L and 0.16 mg/L respectively).

Table 8A. Results and Calculated MDL and ML Values for Interlaboratory Validation Laboratories (mg/L)

	Lab No. 1	Lab No. 5	Lab No. 7	Lab No. 8	Lab No. 9	Lab No. 10
Ref. Water Conc.	0.09	0.09	0.09	0.09	0.09	0.09
Rep. 1	0.10	0.23	0.14	0.20	0.08	0.14
Rep. 2	0.10	0.21	0.17	0.10	0.10	0.13
Rep. 3	0.10	0.20	0.14	0.11	0.11	0.13
Rep. 4	0.09	0.16	0.11	0.09	0.10	0.15
Rep. 5	0.09	0.18	0.21	0.10	0.08	0.13
Rep. 6	0.09	0.18	0.14	0.11	0.07	0.14
Rep. 7	0.09	0.23	0.21	0.12	0.08	0.13
Mean (X_{avg})	0.09	0.20	0.16	0.12	0.09	0.14
Std. Deviation (s)	0.01	0.03	0.04	0.04	0.01	0.01
Student's t value	3.143	3.143	3.143	3.143	3.143	3.143
MDL	0.01	0.08	0.12	0.12	0.05	0.02
ML	0.04	0.27	0.38	0.37	0.15	0.08

Table 8B. Results and Calculated MDL and ML Values for In-house Validation Laboratory, Sets 1-6 (mg/L)

	Lab No. 13	Lab No. 13	Lab No. 13	Lab No. 13	Lab No. 13	Lab No. 13
Replicate Set No.	1	2	3	4	5	6
Ref. Water Conc.	0.07	0.07	0.07	0.07	0.07	0.07
Rep. 1	0.10	0.10	0.09	0.08	0.07	0.06
Rep. 2	0.09	0.10	0.08	0.07	0.06	0.06
Rep. 3	0.10	0.11	0.07	0.07	0.05	0.07
Rep. 4	0.08	0.10	0.09	0.07	0.06	0.06
Rep. 5	0.08	0.09	0.07	0.07	0.05	0.06
Rep. 6	0.07	0.09	0.07	0.07	0.06	0.06
Rep. 7	0.07	0.08	0.07	0.10	0.06	0.07
Mean (X_{avg})	0.08	0.10	0.08	0.08	0.06	0.06
Std. Deviation (s)	0.013	0.010	0.010	0.011	0.007	0.005
Student's t value	3.143	3.143	3.143	3.143	3.143	3.143
MDL	0.04	0.03	0.03	0.04	0.02	0.02
ML	0.13	0.10	0.10	0.11	0.07	0.05

Table 8C. Results and Calculated MDL and ML Values for In-house Validation Laboratory, Sets 7-11 (mg/L)

	Lab No. 13	Lab No. 13	Lab No. 13	Lab No. 13	Lab No. 13
Set No.	7	8	9	10	11
Ref. Water Conc.	0.07	0.07	0.07	0.07	0.07
Rep. 1	0.07	0.09	0.07	0.08	0.08
Rep. 2	0.07	0.07	0.07	0.08	0.07
Rep. 3	0.07	0.06	0.07	0.08	0.07
Rep. 4	0.06	0.07	0.06	0.07	0.07
Rep. 5	0.06	0.06	0.06	0.07	0.07
Rep. 6	0.07	0.06	0.06	0.06	0.07
Rep. 7	0.07	0.06	0.05	0.09	0.07
Mean (X_{avg})	0.07	0.07	0.06	0.08	0.07
Std. Deviation (s)	0.005	0.011	0.008	0.010	0.004
Student's *t* value	3.143	3.143	3.143	3.143	3.143
MDL	0.02	0.03	0.02	0.03	0.01
ML	0.05	0.11	0.08	0.10	0.04

Table 9. Pooled MDL and ML Results (mg/L)

Data Source	Pooled MDL	ML
In-house study	0.02	0.07
Interlaboratory study	0.06	0.18
Pooled	0.05	0.16

## Bench Marking of EPA Winkler and Membrane Methods with Luminescence

In a separate in-house study conducted several months before the interlaboratory validation study began, bench marking of EPA Winkler and Membrane Methods with luminescence was evaluated. Multiple replicates of reference water prepared at theoretical DO concentrations across an order of magnitude were measured. The results indicated the overall accuracy and precision of the DO measurements favored the luminescence procedure (Figures 1 and 2). The greatest bias and precision of the two EPA methods occurred at DO concentrations 3.5 mg/L to 10.3 mg/L, whereas the luminescence was accurate and precise at all tested DO concentrations (1.7 mg/L to 10.3 mg/L). And while an analysis of variance test (ANOVA) for all three DO methods reported statistical indifference, the most notable statistic between the three methods was precision (Figure 2). The luminescence procedure yielded a relative standard deviation (0.07%) three times lower than the membrane method (0.22%) and 20 times lower than the Winkler method (1.4%).

Figure 1. In-house Dissolved Oxygen Measurements by Method to Theoretical Values
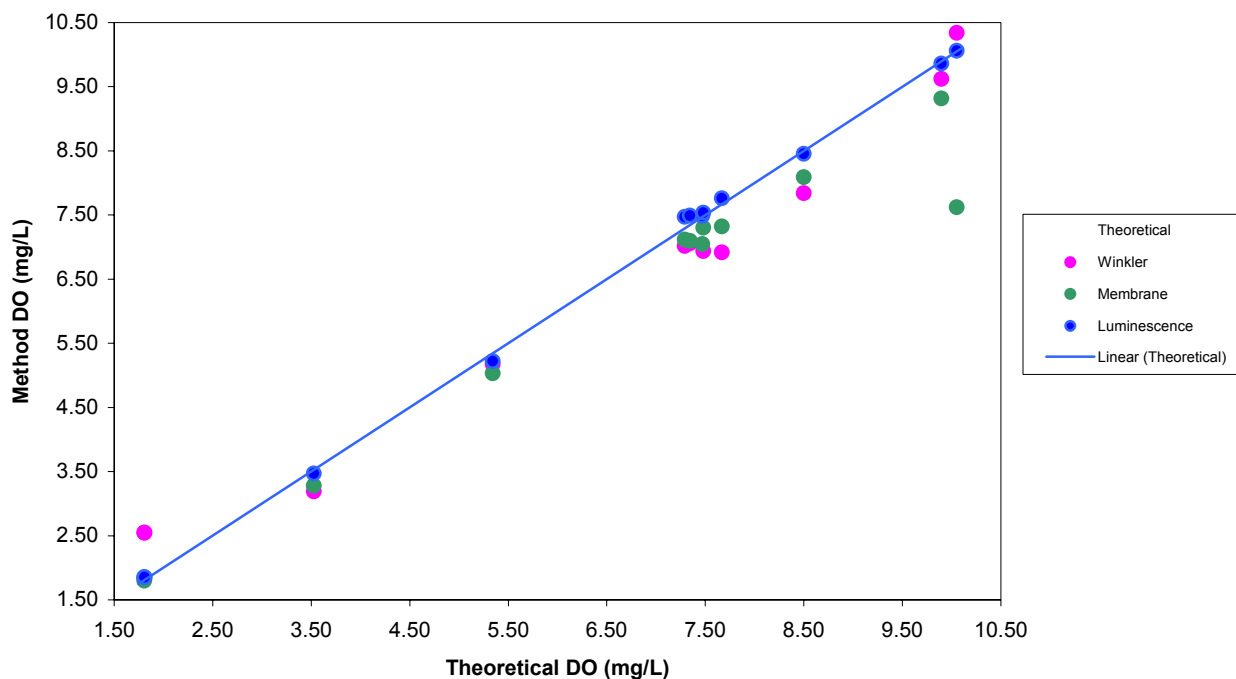
Note – Theoretical DO values (Hitchman, 1978) were determined from measured temperature and pressure values and the precise O_2 concentrations in NIST traceable O_2/N_2 gas mixtures used to prepare the DO reference water samples.

Figure 2. In-house Accuracy and Precision in Air-saturated Water Samples by Method
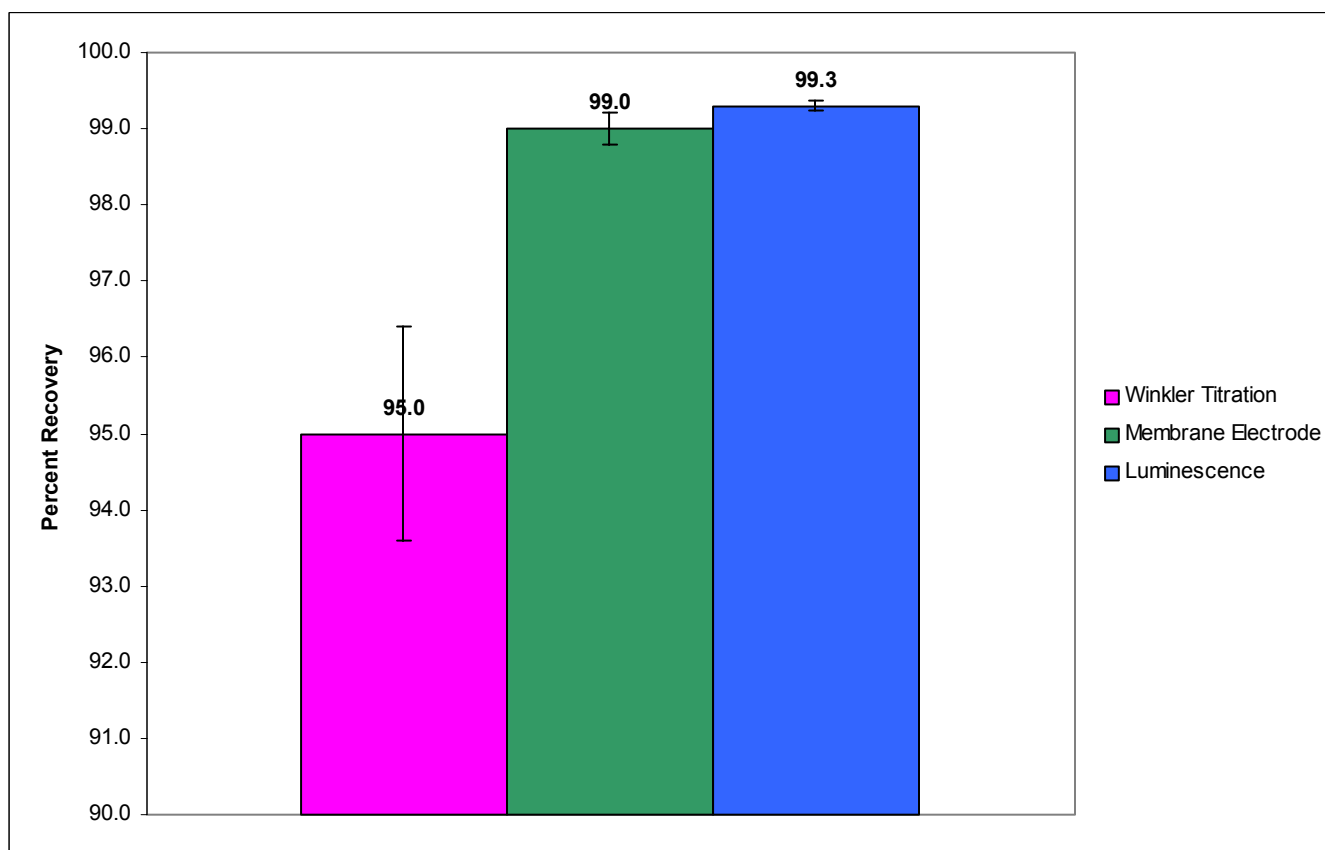
Note – Error bars represent the percent standard deviation about the mean (%RSD). Method %RSD values are 1.4% (Winkler), 0.22% (Membrane), and 0.07% (Luminescence).

The better performing luminescence procedure was further confirmed in the interlaboratory study over two different days with the analysis of air-saturated water (Table 10). Accuracy and precision favored luminescence, with DO recovery being 100% of theoretical concentration and a relative standard deviation of 2.7%. As a result, it was concluded that luminescence is the most accurate and precise procedure for the measurement of DO and that the proceeding comparative methods study with influent-to treatment and final effluent wastewater samples should be compared to that of the better performing luminescence procedure.

Table 10. Interlaboratory Study Air-saturated Water DO Recovery from Calibration Verification Samples

Lab No.	Data Set 1 (Day 0)			Data Set 2 (Day 5)		
	Winkler % Rec.	Membrane % Rec.	LDO % Rec.	Winkler % Rec.	Membrane % Rec.	LDO % Rec.
1	88.2	95.7	96.7	96.1	99.4	96.0
2	97.5	98.9	98.2	97.5	98.9	98.9
3	97.9	99.0	101	98.7	98.9	99.2
5	99.9	98.1	99.2	97.4	97.7	99.5
6	96.7	96.6	100	91.0	90.6	101
7	94.3	94.3	101	94.5	94.5	98.4
8	95.0	96.1	101	96.2	96.2	99.6
9	109	104	107	106	105	104
10	93.2	90.8	98.6	93.8	94.8	94.8
11	104	104	102	105	97.9	102
Average	97.5	97.7	101	97.6	97.4	99.3
Stdev	5.6	4.0	2.9	4.7	3.8	2.7
%RSD	5.8	4.1	2.9	4.8	3.9	2.7
Pooled Data Sets						
Average	97.6	97.5	100			
Stdev	5.0	3.8	2.7			
%RSD	5.2	3.9	2.7			

Comparative Methods with Influent-to-treatment and Final Effluent Wastewater Samples

This study compared the results from the two EPA approved methods (Winkler, and Membrane) with that of the luminescence procedure, using influent-to-treatment and final effluent wastewater samples. Ten of the twelve interlaboratory validation laboratories were used to compare and contrast method results from influent and effluent wastewater samples. Data from the two remaining laboratories were excluded from this study for not following the validation study protocol.

Prior to DO analysis, influent-to-treatment and final effluent wastewater samples were prepared for BOD determination according to Method 5210B (Standard Methods, 20th edition). Two sets of influent and effluent dilutions were prepared in triplicate. Effluent samples received BOD seed. The first set of BOD samples (Day 0) was immediately analyzed then discarded. The second set of samples was incubated for 5 days, and then analyzed.

To compare the performance of each method and procedure in wastewater samples, a single-correlation analysis was performed. The DO results from each of the laboratory side-by-side comparisons with influent-to-treatment and final effluent wastewater (Tables 12A through 12J) were regressed against luminescence to obtain a slope and R^2 value. The slope indicates accuracy (bias) and R^2 indicates the scatter or

precision to luminescence. The correlation results are summarized in Table 11 and plotted in Figures 3A through 3C.

Results from regression analysis indicate Winkler and membrane methods have a negative bias relative to luminescence, regardless of matrix. The Winkler method bias was greatest, being 5%, whereas the membrane method bias was 3%. The precision for both Winkler and membrane methods were equivalent (3%) in influent-to-treatment matrices and favored the membrane method in final effluent matrices (4% vs. 8%) relative to luminescence.

Table 11. Correlation between Luminescence, Winkler, and Membrane for Influent-to-treatment and Final Effluent Wastewater Samples

Method	Comparative Criterion	Influent	Effluent	Influent + Effluent
Winkler	Slope	0.9525	0.9491	0.9506
	(R^2)	0.9736	0.9215	0.9556
Membrane	Slope	0.9637	0.9707	0.9675
	(R^2)	0.9730	0.9647	0.9712

Figure 3A. Dissolved Oxygen Comparison by Method with Influent-to-treatment Wastewater Samples
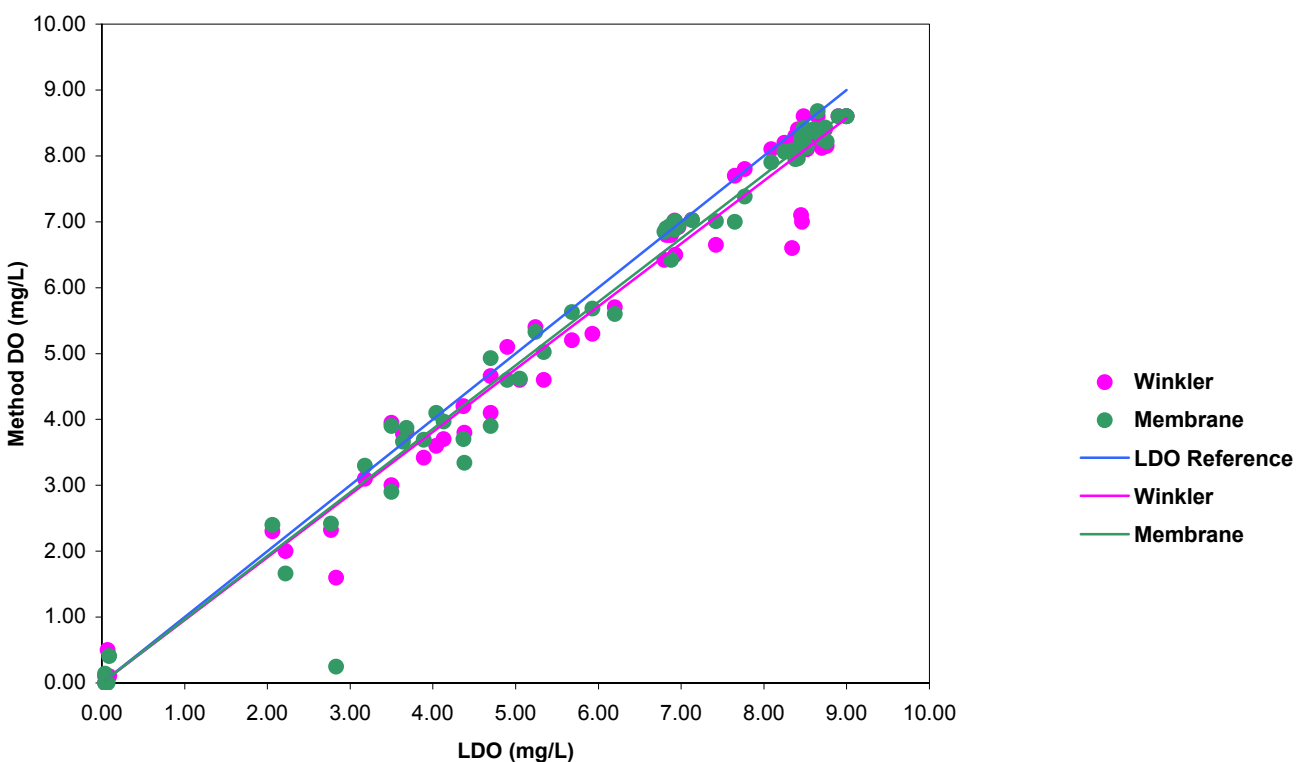
Figure 3B. Dissolved Oxygen Comparison by Method with Final Effluent Wastewater Samples
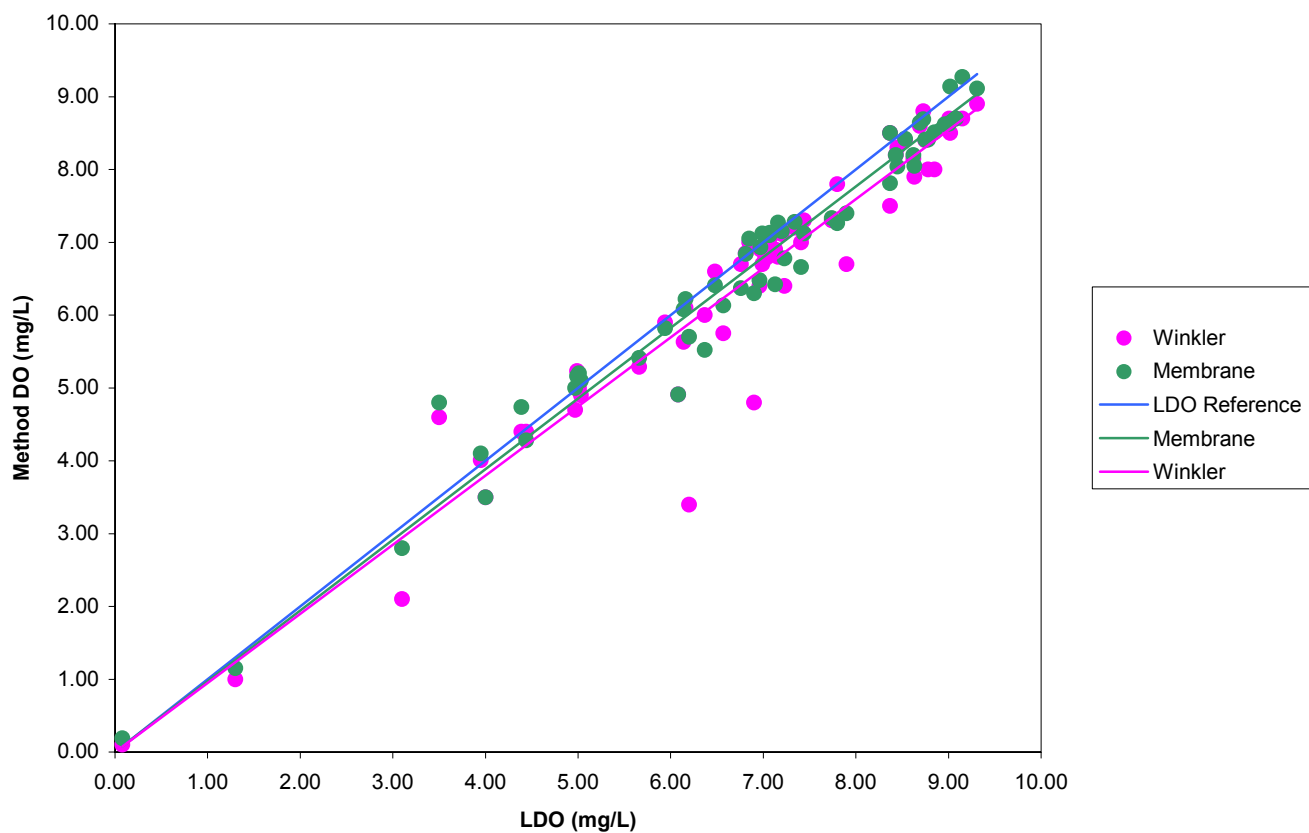
Figure 3C. Dissolved Oxygen Comparison by Method with Combined Data from Influent-to-treatment and Final Effluent Wastewater Samples

Table 12A. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 1
Day 0

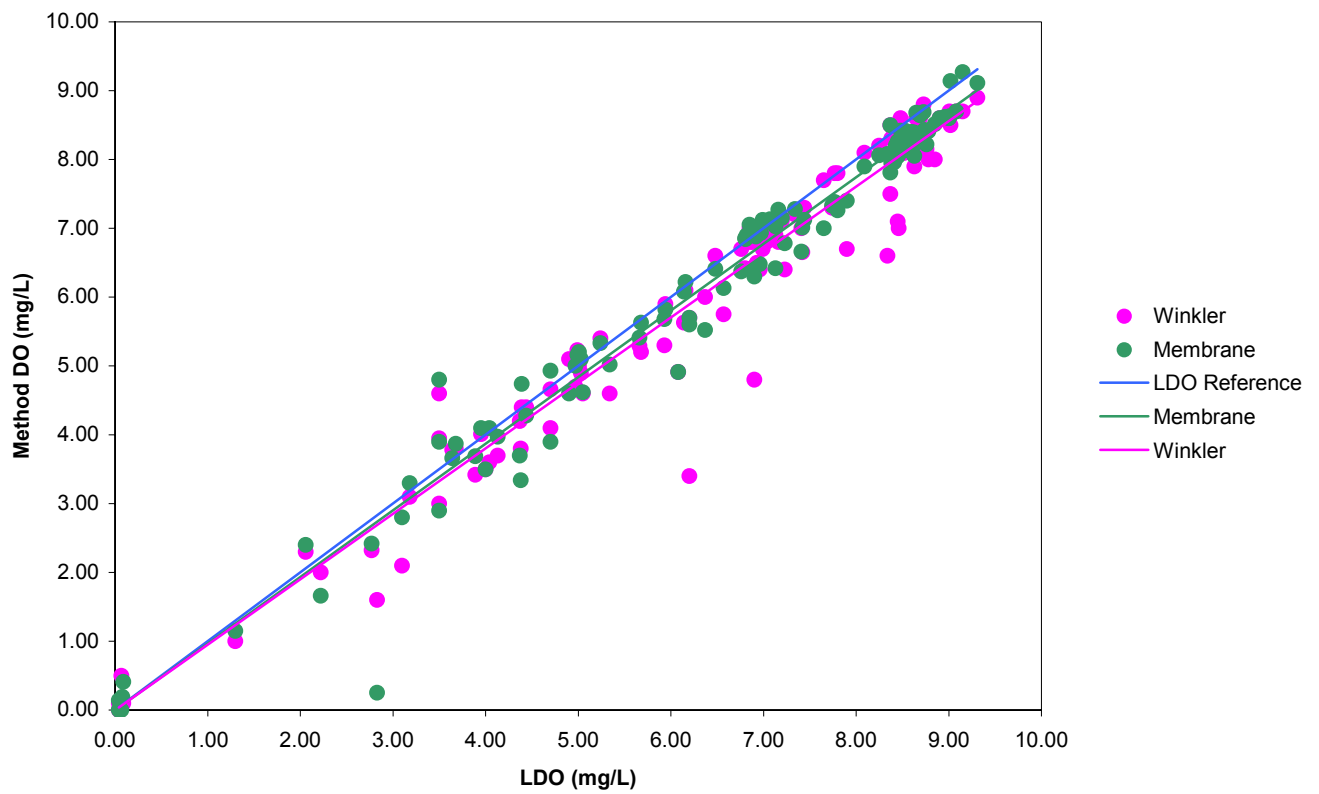| Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	6.50	7.01	6.93		6.4	1.1
Influent Dil B	6.85	6.93	6.86		0.1	1.0
Influent Dil C	6.42	6.85	6.80		5.7	0.7
Average RPD					4.1	1.0
Std. Deviation					3.4	0.21
Effluent Dil A	6.70	7.12	6.99		4.2	1.8
Effluent Dil B	7.05	7.13	7.07		0.3	0.8
Effluent Dil C	6.80	7.27	7.16		5.2	1.5
Average RPD					3.2	1.4
Std. Deviation					2.6	0.51
Calibration Verification % Recovery	88.2	95.7	96.7			

Lab No. 1
Day 5

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	5.20	5.63	5.68		8.8	0.9
Influent Dil B	3.95	3.90	3.50		12.1	10.8
Influent Dil C	2.00	1.66	2.22		10.4	28.9
Average RPD					10.4	13.5
Std. Deviation					1.6	14.2
Effluent Dil A	6.10	6.22	6.16		1.0	1.0
Effluent Dil B	5.63	6.08	6.14		8.7	1.0
Effluent Dil C	5.29	5.41	5.66		6.8	4.5
Average RPD					5.5	2.2
Std. Deviation					4.0	2.0
Calibration Verification % Recovery	96.1	99.4	96.0			

W - Winkler
M – Membrane
L – Luminescence

Table 12B. Individual Laboratory Method Performance with Combined Influent and Effluent Wastewater Samples

Lab No. 2 Day 0						
Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	6.90	7.00	6.91		0.1	1.3
Influent Dil B	6.80	6.90	6.86		0.9	0.6
Influent Dil C	6.80	6.90	6.82		0.3	1.2
Average RPD					0.44	1.0
Std. Deviation					0.39	0.38
Effluent Dil A	6.90	7.10	7.06		2.3	0.6
Effluent Dil B	6.90	7.10	7.07		2.4	0.4
Effluent Dil C	6.80	7.10	7.02		3.2	1.1
Average RPD					2.6	0.71
Std. Deviation					0.48	0.38
Calibration Verification % Recovery	97.5	98.9	98.2			

Lab No. 2 Day 5						
Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	3.60	4.10	4.04		11.5	1.5
Influent Dil B	3.10	3.30	3.18		2.5	3.7
Influent Dil C	2.30	2.40	2.06		11.0	15.2
Average RPD					8.4	6.8
Std. Deviation					5.0	7.4
Effluent Dil A	5.00	5.20	5.01		0.2	3.7
Effluent Dil B	4.90	5.10	5.03		2.6	1.4
Effluent Dil C	4.70	5.00	4.97		5.6	0.6
Average RPD					2.8	1.9
Std. Deviation					2.7	1.6
Calibration Verification % Recovery	97.5	98.9	98.9			

W - Winkler
M – Membrane
L – Luminescence

Table 12C. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 3 Day 0 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	8.10	8.34	8.52		5.1	2.1
Influent Dil B	8.20	8.39	8.63		5.1	2.8
Influent Dil C	8.20	8.40	8.60		4.8	2.4
Average RPD					5.0	2.4
Std. Deviation					0.19	0.35
Effluent Dil A	8.00	8.51	8.85		10.1	3.9
Effluent Dil B	8.00	8.41	8.78		9.3	4.3
Effluent Dil C	8.40	8.41	8.75		4.1	4.0
Average RPD					7.8	4.1
Std. Deviation					3.3	0.21
Calibration Verification % Recovery	97.9	99.0	101			

Lab No. 3 Day 5 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	1.60	0.25[†]	2.83		55.5	168
Influent Dil B	3.80	3.34	4.38		14.2	26.9
Influent Dil C	4.60	5.02	5.34		14.9	6.2
Average RPD					28.2	66.9
Std. Deviation					23.7	87.8
Effluent Dil A	6.00	5.52	6.37		6.0	14.3
Effluent Dil B	6.40	6.48	6.96		8.4	7.1
Effluent Dil C	7.00	6.66	7.41		5.7	10.7
Average RPD					6.7	10.7
Std. Deviation					1.5	2.9
Calibration Verification % Recovery	98.7	98.9	99.2			

[†] - value exceeds lower limit of detection
W - Winkler
M – Membrane
L – Luminescence

Table 12D. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 5 Day 0 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	7.03	7.03	7.13		0.00	1.4
Influent Dil B	6.92	6.94	6.97		0.29	0.72
Influent Dil C	7.02	6.88	6.92		2.0	1.4
Average RPD					1.2	0.81
Std. Deviation					0.41	0.53
Effluent Dil A	6.90	6.93	6.97		1.0	0.58
Effluent Dil B	7.11	7.15	7.20		1.3	0.70
Effluent Dil C	7.20	7.28	7.34		1.9	0.82
Average RPD					1.4	0.70
Std. Deviation					0.47	0.12
Calibration Verification % Recovery	99.9	98.1	99.2			

Lab No. 5 Day 5 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	4.66	4.93	4.70		0.9	4.8
Influent Dil B	3.78	3.66	3.64		3.8	0.5
Influent Dil C	2.32	2.42	2.77		17.7	13.5
Average RPD					7.4	6.3
Std. Deviation					9.0	6.6
Effluent Dil A	5.23	5.16	4.99		1.3	4.7
Effluent Dil B	4.40	4.74	4.39		7.4	0.2
Effluent Dil C	4.01	4.10	3.95		2.2	1.5
Average RPD					3.7	2.1
Std. Deviation					3.3	2.3
Calibration Verification % Recovery	97.4	97.7	99.5			

W - Winkler
M – Membrane
L – Luminescence

Table 12E. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 6 Day 0 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	8.40	8.43	8.74		4.0	3.6
Influent Dil B	8.10	8.11	8.43		4.0	3.9
Influent Dil C	7.80	7.38	7.77		0.4	5.1
Average RPD					2.8	4.2
Std. Deviation					2.1	0.82
Effluent Dil A	8.60	8.62	8.96		4.1	3.9
Effluent Dil B	8.70	8.63	9.01		3.5	4.3
Effluent Dil C	8.70	8.70	9.08		4.3	4.3
Average RPD					4.0	4.15
Std. Deviation					0.41	0.25
Calibration Verification % Recovery	96.7	96.6	100			

Lab No. 6 Day 5 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	3.70	3.97	4.13		11.0	4.0
Influent Dil B	0.10[†]	0.41[†]	0.09		10.5	128
Influent Dil C	0.00[†]	0.14[†]	0.04		200	111
Average RPD					73.8	81.0
Std. Deviation					109	67.3
Effluent Dil A	4.91	4.91	6.08		21.3	21.3
Effluent Dil B	1.00	1.15	1.30		26.1	12.2
Effluent Dil C	0.10[†]	0.19[†]	0.08		22.2	81.5
Average RPD					23.2	38.3
Std. Deviation					2.5	37.6
Calibration Verification % Recovery	92.1	90.6	101			

[†] - value exceeds lower limit of detection
W - Winkler
M – Membrane
L – Luminescence

Table 12F. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 7
Day 0

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	8.12	8.26	8.70		10.9	5.7
Influent Dil B	8.15	8.22	8.76		11.2	4.3
Influent Dil C	8.10	8.13	8.52		12.9	5.3
Average RPD					11.7	5.1
Std. Deviation					1.0	0.71
Effluent Dil A	8.15	8.20	8.62		5.6	5.0
Effluent Dil B	7.90	8.05	8.63		8.8	7.0
Effluent Dil C	7.50	7.81	8.37		11.0	6.9
Average RPD					8.5	6.3
Std. Deviation					2.7	1.1
Calibration Verification % Recovery	94.3	94.3	101			

Lab No. 7
Day 5

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	6.65	7.01	7.42		10.9	5.7
Influent Dil B	5.30	5.68	5.93		11.2	4.3
Influent Dil C	3.42	3.69	3.89		12.9	5.3
Average RPD					11.7	5.1
Std. Deviation					1.0	0.71
Effluent Dil A	7.30	7.33	7.74		5.9	5.4
Effluent Dil B	6.40	6.78	7.23		12.2	6.4
Effluent Dil C	5.75	6.13	6.57		13.3	6.9
Average RPD					10.4	6.3
Std. Deviation					4.0	0.8
Calibration Verification % Recovery	94.5	94.5	98.4			

W - Winkler
M – Membrane
L – Luminescence

Table 12G. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 8 Day 0 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	8.60	8.60	9.00		4.5	4.5
Influent Dil B	8.60	8.60	9.00		4.5	4.5
Influent Dil C	8.60	8.60	8.90		3.4	3.4
Average RPD					4.2	4.2
Std. Deviation					0.64	0.64
Effluent Dil A	6.70	7.40	7.90		16.4	6.5
Effluent Dil B	4.80	6.30	6.90		35.9	9.1
Effluent Dil C	3.40	5.70	6.20		58.3	8.4
Average RPD					36.9	8.0
Std. Deviation					21.0	1.3
Calibration Verification % Recovery	95.0	96.1	101			

Lab No. 8 Day 5 Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	5.70	5.60	6.20		8.4	10.2
Influent Dil B	4.10	3.90	4.70		13.6	18.6
Influent Dil C	3.00	2.90	3.50		15.4	18.8
Average RPD					12.5	15.8
Std. Deviation					3.6	4.9
Effluent Dil A	4.60	4.80	3.50		27.2	31.3
Effluent Dil B	3.50	3.50	4.00		13.3	13.3
Effluent Dil C	2.10	2.80	3.10		38.5	10.2
Average RPD					26.3	18.3
Std. Deviation					12.6	11.4
Calibration Verification % Recovery	96.2	96.2	99.6			

W - Winkler
M – Membrane
L – Luminescence

Table 12H. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 9
Day 0

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	8.60	8.68	8.65		0.58	0.35
Influent Dil B	8.60	8.42	8.48		1.4	0.71
Influent Dil C	8.10	7.90	8.09		0.12	2.4
Average RPD					0.70	1.1
Std. Deviation					0.65	1.1
Effluent Dil A	8.60	8.64	8.69		1.0	0.58
Effluent Dil B	8.40	8.42	8.53		1.5	1.3
Effluent Dil C	8.80	8.69	8.73		0.80	0.46
Average RPD					1.1	0.78
Std. Deviation					0.38	0.45
Calibration Verification % Recovery	109	104	107			

Lab No. 9
Day 5

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	3.81	3.87	3.68		3.5	5.0
Influent Dil B	0.50	0.00[†]	0.07		151	200
Influent Dil C	0.10[†]	0.00[†]	0.04		85.7	200
Average RPD					80.0	135
Std. Deviation					73.9	113
Effluent Dil A	6.85	6.84	6.81		0.59	0.44
Effluent Dil B	5.90	5.82	5.94		0.68	2.0
Effluent Dil C	4.40	4.28	4.44		0.90	3.7
Average RPD					0.72	2.1
Std. Deviation					0.16	1.6
Calibration Verification % Recovery	106	105	104			

[†] - value exceeds lower limit of detection
W - Winkler
M – Membrane
L – Luminescence

Table 12I. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 10
Day 0

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	7.00	8.12	8.46		18.9	4.1
Influent Dil B	7.10	8.26	8.45		17.4	2.3
Influent Dil C	6.60	8.08	8.34		23.3	3.2
Average RPD					19.8	3.2
Std. Deviation					3.1	0.91
Effluent Dil A	8.50	9.14	9.02		5.9	1.3
Effluent Dil B	8.70	9.27	9.15		5.0	1.3
Effluent Dil C	8.90	9.11	9.31		4.5	2.2
Average RPD					5.2	1.6
Std. Deviation					0.72	0.50
Calibration Verification % Recovery	93.2	90.8	98.6			

Lab No. 10
Day 5

Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	5.40	5.33	5.24		3.0	1.7
Influent Dil B	4.60	4.62	5.05		9.3	8.9
Influent Dil C	4.20	3.70	4.37		4.0	16.6
Average RPD					5.4	9.1
Std. Deviation					3.4	7.5
Effluent Dil A	6.90	6.42	7.13		3.3	10.5
Effluent Dil B	6.70	6.37	6.76		0.89	5.9
Effluent Dil C	6.60	6.41	6.48		1.8	1.1
Average RPD					2.0	5.8
Std. Deviation					1.2	4.7
Calibration Verification % Recovery	93.8	94.8	94.8			

W - Winkler
M – Membrane
L – Luminescence

Table 12J. Individual Laboratory Method Performance with Influent and Effluent Wastewater Samples

Lab No. 11 Day 0						
Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	8.40	7.96	8.41		0.12	5.5
Influent Dil B	8.30	7.95	8.38		0.96	5.3
Influent Dil C	8.20	8.06	8.25		0.61	2.3
Average RPD					0.6	4.4
Std. Deviation					0.4	1.8
Effluent Dil A	8.30	8.04	8.45		1.8	5.0
Effluent Dil B	8.20	7.96	8.43		2.8	5.7
Effluent Dil C	8.50	8.10	8.37		1.5	3.3
Average RPD					2.0	4.7
Std. Deviation					0.65	1.2
Calibration Verification % Recovery	104	104	102			

Lab No. 11 Day 5						
Sample	Winkler (mg/L)	Membrane (mg/L)	LDO (mg/L)		W-L RPD	M-L RPD
Influent Dil A	7.70	7.00	7.65		9.5	0.65
Influent Dil B	6.80	6.42	6.88		5.7	1.2
Influent Dil C	5.10	4.60	4.90		10.3	4.0
Average RPD					8.5	1.9
Std. Deviation					2.4	1.8
Effluent Dil A	7.80	7.26	7.80		0.00	7.2
Effluent Dil B	7.30	7.12	7.44		1.9	4.4
Effluent Dil C	7.00	7.05	6.85		2.8	2.9
Average RPD					1.4	4.8
Std. Deviation					1.2	2.2
Calibration Verification % Recovery	105	97.9	102			

W - Winkler
M – Membrane
L – Luminescence

The DO results from each of the two EPA methods in Tables 12A through 12J were then evaluated with respect to luminescence, using a differentiating model of relative percent difference (RPD). The RPD$_{avg}$ values for influent-to-treatment and final effluent samples are summarized in Tables 13A through 13C.

The results of RPD analysis further indicate both Winkler and membrane methods are biased relative to the luminescence procedure. The Winkler method bias was greatest (7%) followed by the membrane method (5%), both, regardless of matrix. Similarly, Winkler had the greatest imprecision (8%) followed by the membrane method (4%).

Table 13A. Summary Performance of each Method with Influent-to-treatment Wastewater Samples

Lab No.	Day 0		Day 5	
	RPD$_{avg}$ WDO-LDO	RPD$_{avg}$ MDO-LDO	RPD$_{avg}$ WDO-LDO	RPD$_{avg}$ MDO-LDO
1	4.1	1.0	10.4	13.5
2	0.44	1.0	8.4	6.8
3	5.0	2.4	28.2	66.9[†]
5	1.2	0.81	7.4	6.3
6	2.8	4.2	73.8[†]	81.0[†]
7	6.4	5.4	11.7	5.1
8	4.2	4.2	12.5	15.8
9	0.70	1.1	80.0[†]	135[†]
10	19.8	3.2	5.4	9.1
11	0.56	4.7	1.9	7.4
Mean	4.5	2.8	10.7[‡]	9.1[‡]
Std. Deviation	5.8	1.7	7.9[‡]	4.0[‡]
Pooled Multiple Days	RPD$_{avg}$ WDO-LDO		RPD$_{avg}$ MDO-LDO	
Mean	7.28[‡]		5.39[‡]	
Stdev.	7.29[‡]		4.26[‡]	

[†] Calculation included unreliable DO values from Winkler and membrane methods that exceeded lower limit of detection
[‡] Calculation does not include [†] data
RPD$_{avg}$ – averaged RPD from three dilution DO values
WDO – Winkler Dissolved Oxygen
MDO – Membrane Dissolved Oxygen
LDO – Luminescence Dissolved Oxygen
Stdev. – Standard deviation

Table 13B. Summary Performance of each Method with Final Effluent Wastewater Samples

Lab No.	Day 0		Day 5	
	RPD_{avg} WDO-LDO	RPD_{avg} MDO-LDO	RPD_{avg} WDO-LDO	RPD_{avg} MDO-LDO
1	3.2	1.4	5.5	2.2
2	2.6	0.71	2.8	1.9
3	7.8	4.1	6.7	10.1
5	1.4	0.70	2.1	4.9
6	4.0	4.2	23.2[†]	38.3[†]
7	8.5	6.3	10.4	6.3
8	36.9	8.0	26.3	18.3
9	1.1	0.78	0.72	2.1
10	5.2	1.6	2.0	5.8
11	2.0	3.1	1.4	4.8
Mean	7.3	3.1	6.4[‡]	6.3[‡]
Std. Deviation	10.7	2.5	8.1[‡]	5.2[‡]
Pooled Multiple Days	RPD_{avg} WDO-LDO		RPD_{avg} MDO-LDO	
Mean	6.89[‡]		4.59[‡]	
Stdev.	9.29[‡]		4.24[‡]	

[†] Calculation included unreliable DO values from Winkler and membrane methods that exceeded lower limit of detection
[‡] Calculation does not include [†] data
RPD_{avg} – averaged RPD from three dilution DO values
WDO – Winkler Dissolved Oxygen
MDO – Membrane Dissolved Oxygen
LDO – Luminescence Dissolved Oxygen
Stdev. – Standard deviation

Table 13C. Summary Performance of each Method Combined with Multiple Day Results and Influent-to-treatment and Final Effluent Wastewater Results

Lab No.	RPD_{avg} WDO-LDO	RPD_{avg} MDO-LDO
Mean RPD_{avg}	7.07[‡]	4.92[‡]
Stdev.	8.27[‡]	4.16[‡]

[‡] Calculations do not include [†] data from Tables 11A and 11B
RPD_{avg} – averaged RPD from three dilution DO values
WDO – Winkler Dissolved Oxygen
MDO – Membrane Dissolved Oxygen
LDO – Luminescence Dissolved Oxygen
Stdev. – Standard deviation

To further support the validity of the comparative influent-to treatment and final wastewater statistical interpretation, the interlaboratory validation calibration verification results of Winkler and membrane methods and the luminescence procedure (Tables 12 through 12J) were statistically averaged and normalized to obtain a percent relative standard deviation (Table 10). The relative standard deviation indicates the relative error, or how well the result agrees to the theoretical DO concentration. Results of

Winkler and membrane calibrations were within 97.5% and luminescence at 100% of the theoretical DO concentration. This indicates each method was properly calibrated and verified against theoretical DO (Hitchman, 1978), and that measured bias precision from Winkler and membrane in wastewater is due solely to the intrinsic bias of the method determinant and sample matrix interferences (Table 14).

Table 14. Summary of Bias and Precision for Winkler Titration, Membrane Probe, and Luminescence Sensor Methods

Method	Reference Water[†]		POTW Wastewater[‡]	
	%Bias$_{avg}$	%Precision$_{avg}$	%Bias$_{avg}$	%Precision$_{avg}$
Winkler	2.5	5.0	5.0	4.4
Membrane	2.5	3.8	3.3	2.9
Luminescence	0.0	2.5	0.0[⨽]	2.5[⨽]

[†] - Interlaboratory validation study of air-saturated water calibration verification

[‡] - Regression analysis (Table 11)

[⨽] - By inference from Hach Company in-house and interlaboratory validation study of air-saturated water calibration verification

Section 7
General Discussions and Conclusions

Twelve POTW laboratories from the states of California, Colorado, Florida, Illinois, Missouri and Texas participated in the interlaboratory validation study. Out of these 12 laboratories, the data from 7 laboratories was used for determining IPR_{low} QC performance criteria, 10 laboratories for IPR_{high} QC performance criteria, 6 laboratories for MDL and ML QC performance criteria, and 10 laboratories for wastewater matrix methods comparison and correlation.

Initial precision and recovery studies were conducted at two different concentration level ranges (IPR low $_{1.72 - 1.74\ mg/L}$ and IPR high $_{7.22 - 9.23\ mg/L}$) to bracket the DO reporting range of BOD Method 5210B (*Standard Methods*, 20th ed). Statistical results indicate both IPR tests have a mean recovery of 100%, with less than a 0.7% point difference in precision (1.75% and 1.10%, respectively). From these results, it can be concluded that nearly all laboratories are capable of performing acceptable IPR results successfully, and that the IPR QC acceptance criteria are realistic and reflect performance of the proposed luminescence method. However, due to the high precision of the luminescence measurement, which is atypical of other EPA regulatory precision limits, the calculated 95th upper limit precision criteria should be expanded to reflect reasonable data quality objectives.

Eleven MDL data sets from the in-house study and six data sets from the interlaboratory validation study were used to develop pooled MDL (0.05 mg/L) and ML (0.16 mg/L) values. Justification to include the in-house MDL data (0.02 mg/L) was based on the results of the validation laboratories' analytical precision results from 0.09 mg/L reference water samples (pooled MDL of 0.06 mg/L), and on the reasoning that the development and validation of a method should be representative of a better performing laboratory. The better performing in-house laboratory results were based on a significant amount experience and laboratory technique with the luminescence method. In contrast, the validation laboratories had relatively little experience with the luminescence method prior to the analysis of the MDL reference water samples. Here, each laboratory had performed only two luminescence DO measurements in air-saturated water (Phase I of the interlaboratory study). Therefore, it was concluded that with some practice, each laboratory performing the proposed luminescence method could achieve the pooled MDL of 0.05 mg/L.

Based on an earlier Hach Company in-house study evaluating the accuracy and precision of the Winkler titration, membrane probe methods, and the luminescence procedure over a wide range of theoretical (Hitchman, 1978) DO concentrations, it was concluded that the luminescence procedure was the most accurate (99.3% recovery and precise (0.22% RSD) method (Figures 5). This conclusion was further substantiated by the interlaboratory validation study with individual method calibration verification results (Table 10). Pooled calibration verification data for each method, from two different days of the validation study, yielded a Winkler titration and membrane probe bias of approximately 3%. As a result, differentiation (RPD) and correlation

(regression) statistics from influent-to-treatment and final effluent wastewater samples with Winkler titration and membrane methods were compared to the more accurate and precise luminescence procedure. With respect to influent-to-treatment and final effluent wastewater matrices, both statistical models (Tables 11 and 13A through 13C) indicate that Winkler titration (EPA 360.2) and membrane probe methods (EPA 360.1) are negatively biased relative to that of luminescence, across broad range of DO concentrations.

In summary, this study clearly demonstrates through validation of QC performance reference water samples and POTW wastewater matrices, that the proposed EPA Method 360.3 (luminescence) is superior to the two EPA approved methods (EPA Method 360.1 and EPA Method 360.2). The primary benefit of the luminescence method is better performance with respect to accuracy (bias) and precision, regardless of wastewater matrix (influent-to-treatment and final effluent). Additional benefits to the luminescence method include elimination of toxic and or hazardous chemicals, minimal instrument maintenance, simplicity of use, and increased sample-throughput.

Section 8
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Appendix A
Example of Computations

Initial Precision and Recovery (low level)

1) Mean recovery across laboratories – sum of data points divided by number of data points per laboratory

2) S_b - Standard deviation of mean % recoveries across laboratories

3) S_w – Pooled within standard deviation
Square root of the average variances (standard deviation squared) from each laboratory's four IPR results.

$$\sqrt{1.71}$$

4) S_c – Standard deviation of variability and estimation of mean

$$\sqrt{\left(1+\frac{1}{18}x1.43^2\right)+1.31^2}$$

5) 97.5% lower limit of recovery - mean recovery across laboratories minus Student's *t* value for 18 degrees of freedom (2.1) times S_c.

$$99.5-\left(2.1\,x\,1.96\right)$$

6) 97.5% lower limit of recovery - mean recovery across laboratories plus Student's *t* value for 23 degrees of freedom (2.1) times S_c.

$$99.5+\left(2.1\,x\,1.96\right)$$

7) 95% upper limit of precision – RSD (S_w dived by mean times100) times the square root of the 95th percentile F value with 18 degrees of freedom in the numerator and 54 degrees of freedom in the denominator.

$$1.3166\,x\,100\,x\,\sqrt{1.7770}$$

Initial Precision and Recovery (High level)

1) Mean recovery across laboratories – sum of data points divided by number of data points per laboratory

2) S_b - Standard deviation of mean % recoveries across laboratories

3) S_w – Pooled within standard deviation
Square root of the average variances (standard deviation squared) from each laboratory's four IPR results.

$$\sqrt{0.64}$$

4) S_c – Standard deviation of variability and estimation of mean. The denominator of 3 comes from the average number of data points from each laboratory (validation labs had 2 data points and in-house lab had 4 data points).

$$\sqrt{\left(1+\frac{1}{23}x1.74^2\right)+\left(\frac{1}{4}-\frac{1}{3}x\right)x0.83^2}$$

5) 97.5% lower limit of recovery - mean recovery across laboratories minus Student's *t* value for 23 degrees of freedom (2.1) times S_c.

$$99.5-\left(2.1\,x\,1.76\right)$$

6) 97.5% lower limit of recovery - mean recovery across laboratories plus Student's *t* value for 23 degrees of freedom (2.1) times S_c.

$$99.5+\left(2.1\,x\,1.76\right)$$

7) 95% upper limit of precision – RSD (S_w dived by mean times100) times the square root of the 95th percentile F value with 23 degrees of freedom in the numerator and 46 degrees of freedom in the denominator.

$$0.0083\,x\,100\,x\,\sqrt{1.7630}$$

Method Detection Limit and Method Limit

<u>Laboratory 1</u>

1) MDL – standard deviation of the mean recoveries of 7 replicate analyses of MDL reference water times the Student's *t* value for 6 degrees of freedom

$$0.0049 \, x \, 3.143$$

2) ML – 3.18 times the MDL

$$3.18 \, x \, 0.01$$

3) Pooled MDL – Square root of the average laboratory MDL values squared times the ratio of Student's *t* values with infinite degrees of freedom in the numerator and 16 degrees of freedom in the denominator

$$\sqrt{0.0029} \, x \, \frac{2.32635}{2.58349}$$

Relative Percent Difference

<u>Laboratory 10</u>

Influent Dil. A Day 0

Membrane   Luminescence
8.12 mg/L   8.46mg/L

$$RPD = \frac{|8.12 - 8.46|}{\frac{8.12 + 8.46}{2}} \, x \, 100$$